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Antiproliferative and antimetastatic properties of 3-benzyloxy-16-hydroxymethylene-estradiol analogs against breast cancer cell lines

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ABSTRACT

Despite emerging new therapeutic opportunities, cancer is still a major health problem and a leading cause of death worldwide. Breast tumors are the most frequently diagnosed female malignancies, and the triple-negative subtype is associated with poorer prognosis and lower survival rates than other breast cancer types. The aims of the present study were to determine the anticancer potency of a set of C-3 and C-16 modified estradiol-derivatives against a panel of breast cancer cell lines, and to characterize the mechanism of action of two selected compounds (**1** and **5**) against the MDA-MB-231 triple-negative breast cancer cell line. Growth-inhibitory properties were investigated by an MTT-assay. Cell cycle analysis by flow cytometry has revealed G1 phase accumulation and indicated the proapoptotic effect of **1** and **5** through the elevation of the apoptotic subG1 phase on MDA-MB-231 cells after 24 h treatment. The antimetastatic activities of these compounds were examined by wound healing and Boyden chamber assays, and both compounds were shown to significantly inhibit the migration and invasion of MDA-MB-231 cells at sub-antiproliferative concentrations. Gelatin zymography assay has indicated that matrix metalloproteinase-2 and -9 are not involved in the antimetastatic action of the molecules. Western blot analysis was performed with 24 h incubation to examine the possible changes in the level of focal adhesion kinase (FAK), and both compounds were found to inhibit the phosphorylation of FAK in a concentration-dependent manner in MDA-MB-231 cells. The results of this study demonstrate that C-3 and C-16 modified estradiol derivatives are potent antiproliferative and antimetastatic compounds against a triple-negative breast cancer cell line with a mechanism of action involving the inhibition of FAK, a novel anticancer therapeutic target. Therefore, these findings can be utilized in the development of promising anticancer agents with steroid skeleton.

1. Introduction

Cancer is a crucial cause of morbidity and mortality worldwide, with the incidence of malignant diseases rising both in highly developed and less developed countries. In 2012, 14.1 million new patients and 8.2 million cancer-related deaths were estimated globally, and > 3.14 million new cancer cases were diagnosed in Europe. Amongst women, the most common cancer type is breast cancer, characterized by the highest incidence and mortality rates, comprising 25% of all diagnosed cancer cases and causing 15% of all cancer-related deaths (Steliarova-Foucher et al., 2015; Torre et al., 2015).

Triple-negative breast cancer (TNBC) is a subtype of breast tumors described by the downregulation of estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2). Approximately 15–20% of all breast cancers are triple-negative tumors, and this subtype is responsible for 25% of breast cancer-related deaths (Lee and Djamgoz, 2018). TNBC patients are typically 5–10 years younger than other breast cancer patients at the time of diagnosis (Newman et al., 2015). Indicators for the aggressive behavior of TNBC include a high recurrence rate and faster metastatic spread, leading to poorer prognosis amongst all breast cancer subtypes. Regarding recurrence, the secondary tumor usually occurs within 5 years from the initial diagnosis, with the brain and viscera being affected more often than in case of non-TNBC primaries which usually cause

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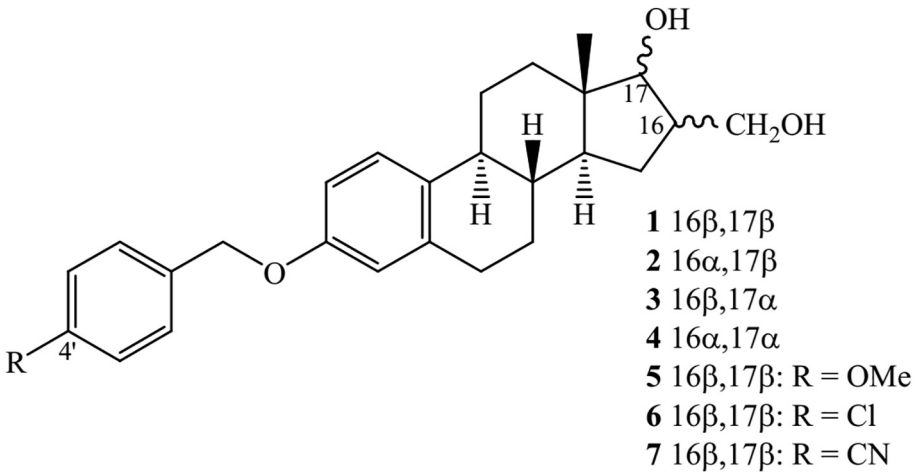

Fig. 1. Chemical structures of the synthesized and investigated estradiol analogs.

metastasis to bone. Compared to other subtypes, TNBC has a shorter median time to death (4.2 vs. 6 years) and worse overall survival. Both of these basic triple-negative features and the possible molecular diversity within TNBCs hamper the development of targeted therapies. Although several novel pharmacological groups of drugs, like poly (ADP-ribose) polymerase inhibitors and immunotherapeutic agents have shown promising results in clinical trials, developing specified treatment for TNBC is still an urgent unmet medical need (Lee and Djamgoz, 2018; Lin et al., 2008).

Estrogens have an essential influence on the evolution of reproductive processes. Furthermore, they play a role in the development of hormone-dependent tumors of the reproductive organs, such as breast, ovarian, endometrial, uterine and prostate cancers (Ascenzi et al., 2006; Chen et al., 2008; Nelles et al., 2011; Pearce and Jordan, 2004). Although the proliferation-stimulating activity of 17β-estradiol is well-established, numerous studies have also investigated the anticancer potential of structurally modified estrogen derivatives (Bodnár et al., 2016; Cadot et al., 2007; Cushman et al., 1997; Jourdan et al., 2011; Mernyák et al., 2014; Mernyák et al., 2015; Möller et al., 2009; Szabó et al., 2016).

A fundamental requirement for estrane-based anticancer drug candidates is the lack of their hormonal activity. Certain structural modifications, such as substitution at C-2 or C-3 of the estrane skeleton lead to the loss of the estrogenic properties of the compounds (Anstead et al., 1997; Cushman et al., 1997; Wölfling et al., 2003). Additionally, modification of the D-ring of the estrange skeleton leading to D-homo and D-seco derivatives results complete loss of estrogenic activity (Jovanovic-Santa et al., 2003; Jovanovic-Santa et al., 2015). Moreover, some of such agents may additionally interact with the enzyme aromatase (CYP1A19) indicating a secondary way of intervention in the estrogen-dependent proliferative disorders including gynecological cancers, endometriosis (Trifunovic et al., 2017).

In a previous study, the binding abilities of possible isomers of 16-hydroxymethyl-3,17-estradiol to estrogen receptor were investigated by radioligand-binding assay, utilizing receptor protein prepared from rabbit uteri. The relative binding affinities (RBA) of each estradiol derivative was lower than 2.0% of that of estradiol, indicating that the introduction of a 16-hydroxymethyl group abolishes the affinity independently of its configuration (Tapolcsányi et al., 2002). Consequently, the presence of a hydroxymethylene group at C-16 is detrimental concerning the estrogenic activity of the tested compounds. Based on these preliminary findings, it can be stated that the 16-hydroxymethylene-3,17-estradiol core might be suitable for the design of biologically active estrone derivatives lacking hormonal activity.

We have recently reported that the presence of a 3-benzyl ether function on the estrane core may enhance the antiproliferative potential of the steroid (Mernyák et al., 2014; Mernyák et al., 2015). In order to combine the structural moieties responsible for an increase in the antiproliferative and a decrease in the estrogenic actions, here we have synthesized additional 3-benzyl ethers bearing electronically different substituents at the 4′-position.

The aim of the current investigation was to characterize the anticancer properties of these 16-hydroxymethyl-estradiol analogs on a panel of breast cancer cell lines in vitro. The most promising agents were selected for further analyses in order to describe the mechanism of the action against the MDA-MB-231 triple-negative breast cancer cell line. Although the hormonal receptor status of the utilized cell lines was different we aimed to describe hormone-independent antiproliferative properties. The effects of the selected molecules on cancer cell migration were also evaluated.

2. Materials and methods

2.1. Chemicals

The four possible stereoisomers of 3-benzyloxy-16-hydroxymethylene-estra-1,3,5(10)-trien-17-ol (**1**–**4**) were synthesized and characterized as reported previously (Tapolcsányi et al., 2002). Three further analogs with a substituted benzyl function at C-3 have also been prepared (**5**–**7**). The synthesis and chemical characterization of the molecules are available as Supplementary material (Fig. 1).

2.2. Cell cultures

Human breast cancer cell lines, including MCF7, T47D, MDA-MB-231 and MDA-MB-361, as well as non-cancerous fibroblasts (MRC-5) were purchased from the European Collection of Authenticated Cell Cultures (ECACC, Salisbury, UK). All cell lines were maintained in minimal essential medium (MEM) supplemented with 10% fetal bovine serum, 1% non-essential amino acids and 1% antibiotic-antimycotic mixture, and were stored in humidified air containing 5% CO_2 at 37 °C. All utilized components were obtained from Lonza Group Ltd. (Basel, Switzerland).

2.3. Determination of antiproliferative activities

To determine the antiproliferative action of the tested compounds against four malignant breast cancer cell lines (MCF-7, T47D, MDA-MB-231 and MDA-MB-361), an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was carried out. Cells were seeded at a density of 5000 cells/well into 96-well plates (in the case of MDA-MB-361 10,000 cells/well), treated with increasing concentrations of 16-

hydroxymethyl-estranes (0.1–30.0 μM) under cell culturing conditions for 72 h. At the end of incubation, 5 mg/ml MTT solution was added to the samples. Precipitated formazan crystals were solubilized in dimethyl sulfoxide and absorbance was measured at 545 nm with a microplate reader. Wells containing untreated cells were used as control (Mosmann, 1983).

Based on these results, sigmoidal dose-response curves were fitted and IC_{50} values were calculated using GraphPad Prism 5.01 (GraphPad Software, San Diego, CA, USA). The growth-inhibitory effects of the tested compounds were compared to cisplatin (Ebewe Pharma GmbH, Unterach, Austria) in the same concentration range. MRC-5 human fibroblasts were also examined by the same method to verify the selectivity of the test compounds towards cancer.

2.4. Cell cycle analysis by flow cytometry

Cellular DNA content was determined by flow cytometry. MDA-MB-231 cells were seeded into 6-well plates at a density of 250,000–300,000 cells/well. After 24 h incubation cells were washed with phosphate-buffered saline (PBS) and gathered with trypsin. The samples were centrifuged at 1500 rpm for 10 min, additionally washed and centrifuged again, and fixed in 1 ml of 70% ethanol, −20 °C, for at least 30 min. The dye solution (0.1 mg/ml PI, 0.003 μl/ml Triton-X, 0.02 mg/ml RNase A, and 1.0 mg/ml sodium citrate dissolved in distilled water) was then added to the samples for 60 min. The cell cycle analysis was carried out by a Partec CyFlow instrument (Partec GmbH, Münster, Germany). In all measurements, 20,000 cells were analyzed. The percentages of cells in different cell cycle phases (subG1, G1, S and G2/M) were determined by the ModFit LT software (Verity Software House, Topsham, ME, USA). The subG1 fraction was considered as the apoptotic cell population (Vermes et al., 2000).

2.5. Wound healing assay

The action of the selected compounds on cell migration was assessed by a wound healing assay. The assay was performed with specific wound assay chambers (ibidi GmbH, Martinsried, Germany). MDA-MB-231 cells were trypsinized and 25,000 cells were seeded into each chamber of the inserts. After an overnight incubation for appropriate cell attachment at 37 °C under 5% CO_2, the culture insert was removed. A washing step with PBS was performed to remove non-adherent cells and debris. Cells were treated with increasing concentrations of each test compound in a medium containing 2% FBS for 24 h. Migration of the cells to the wound site was visualized by a phase-contrast inverted microscope (Axiovert 40, Zeiss, Thornwood, NY, US). Images were taken by a CCD camera at planned intervals to assess wound closure. The rate of migration was calculated as the ratio of wound closure in treated samples after 24 h and 0 h, respectively, as compared with the ratio of the wound closure in untreated control samples after 24 h and 0 h, respectively (Bózsity et al., 2017).

2.6. Boyden chamber assay

Invasion ability of MDA-MB-231 cells was investigated by a BD BioCoat Matrigel Invasion Chamber (BD Biosciences, Bedford, MA, USA) containing an 8 μm pore size PET membrane and a thin layer of matrigel basement matrix. Fifty thousand cells were suspended in serum-free medium and were injected into the top insert before the tested compounds were added in sub-antiproliferative concentrations. A medium containing 10% FBS was used as chemoattractant in the bottom well. The inserts were removed after incubation for 24 h at 37 °C under 5% CO_2 and the upper surface of the membrane was cleaned from non-invading cells with a cotton swab. After a washing step with PBS, the insert was fixed with cold 100% methanol, washed with PBS again and stained with crystal violet solution. The invading and stained cells were counted under a phase-contrast inverted microscope. To determine the rate of invasion, the number of treated samples and untreated controls were counted and compared to each other.

2.7. Gelatin zymography assay

A gelatin zymography assay was utilized to determine how the test compounds affect the activities of matrix metalloprotease-2 (MMP-2) and −9 (Snoek-van Beurden and Von den Hoff, 2005). MDA-MB-231 cells were seeded in experimental dishes at approximately 80% confluency and were allowed to grow overnight. After a washing step with PBS the test compounds were added in serum-free medium and incubated for 24 h. Then the supernatant containing the secreted MMP-2 and MMP-9 enzymes were collected from above the cells and was centrifuged at 6000 rpm at 4 °C for 2 min, then collected again. Equal amounts of the samples mixed with bromophenol blue dye in 5:1 ratio were separated by SDS-PAGE containing 0.1% gelatin via electrophoresis at 120 V for 90 min. Major forms of MMPs were identified by Prism Ultra Protein Ladder molecular size marker (Abcam Cambridge, U.K.). The gel was washed twice by 2.5% Triton X-100 solution, then treated with the reaction solution containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl and 5 mM $CaCl_2$. After incubation for 24 h the gel was stained with Coomassie brilliant blue G-250 (SimplyBlue™ Safestain, Thermo Fisher Scientific, Waltham, MA, USA) for 60 min and washed by distilled water twice. Finally, the gel was captured by a Luminescent Image Analyzer System (LAS 4000 mini, Fujifilm, Tokyo, Japan).

2.8. Western blot assay

A Western Blot assay was performed to evaluate the antimetastatic properties of the examined compounds. Two million cells were seeded in small experimental dishes, and the tested compounds were added in medium containing 2% FBS the next day. Cells were detached from the dishes by a rubber policeman and centrifuged at 7000 rpm, 4 °C for 2 min, washed with PBS and centrifuged again. Whole cell extracts (WCEs) were prepared by re-suspending cells in TEGN lysis buffer (10 mM Tris pH 7.5, 1 mM EDTA, 420 mM NaCl, 10% glycerol and 0.5% NP40) supplemented with 1 mM DTT and a cocktail of protease and phosphatase inhibitor (Roche Applied Science, Penzberg, Germany). After 10 min on ice and centrifugation (13,000 rpm, 4 °C, 10 min) the protein content of the supernatant was mixed with bromophenol blue dye in 2:1 ratio. Equal amounts of WCEs were first separated by SDS-PAGE with electrophoresis for 1 h at 160 V, transferred onto nitrocellulose membranes for overnight at 20 V, 4 °C. Afterwards, the samples were blocked and incubated with a primary antibody followed by an appropriate secondary antibody. The antibodies utilized were FAK pY397 (Becton Dickinson, Franklin Lakes, NJ, USA), FAK (Cell Signaling Technology, Danvers, MA, USA), β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and GAPDH (Cell Signaling Technology). Blots were visualized by enhanced chemiluminescence (ECL-Plus, GE Healthcare Life Sciences, Little Chalfont, UK) using a Luminescent Image Analyzer System.

2.9. Statistical analysis

GraphPad Prism 5.01 was used for the statistical evaluation of measured data in each experiment. To determine statistical significance, one-way ANOVA with Dunnett post-test for comparison of multiple groups was utilized. A p value < 0.05 was regarded as statistically significant.

3. Results

3.1. Antiproliferative activity of the tested compounds

Several 16-hydroxymethyl-estranes proved to be potent

Table 1
Antiproliferative effects of the tested compounds (**1–7**) on human breast cancer cell lines and MRC-5.

		Growth inhibition (%) ± SEM [calculated IC_{50} value (μM)[a]]				
		MDA-MB-231	MCF7	T47D	MDA-MB-361	MRC-5
1	10 μM	31.52 ± 1.82	60.16 ± 1.56	54.49 ± 0.27	93.07 ± 0.52	78.02 ± 2.63
	30 μM	94.90 ± 0.46	97.20 ± 0.41	89.95 ± 043	93.25 ± 1.09	96.50 ± 0.20
	IC_{50} (μM)	12.70	8.15	8.41	4.60	6.73
2	10 μM	93.82 ± 0.49	91.54 ± 0.56	82.73 ± 1.63	93.80 ± 0.70	94.78 ± 0.49
	30 μM	96.21 ± 0.34	97.83 ± 0.24	82.10 ± 0.50	93.98 ± 0.26	96.88 ± 0.21
	IC_{50} (μM)	3.91	5.05	4.58	3.81	5.53
3	10 μM	76.41 ± 0.65	27.02 ± 1.28	38.76 ± 2.02	62.73 ± 2.20	39.10 ± 3.23
	30 μM	92.17 ± 0.59	88.57 ± 1.23	90.29 ± 0.58	86.15 ± 0.93	92.63 ± 0.15
	IC_{50} (μM)	5.93	13.57	15.73	7.21	10.20
4	10 μM	84.34 ± 1.22	56.64 ± 2.23	47.49 ± 2.41	79.06 ± 2.70	53.67 ± 3.39
	30 μM	92.79 ± 0.26	86.85 ± 0.89	94.56 ± 0.73	89.85 ± 1.02	76.84 ± 2.19
	IC_{50} (μM)	5.47	9.05	10.44	5.38	7.33
5	10 μM	89.54 ± 0.82	96.46 ± 0.65	95.58 ± 0.26	81.08 ± 1.77	76.50 ± 2.28
	30 μM	87.73 ± 0.32	96.68 ± 0.17	95.26 ± 0.90	86.97 ± 1.26	79.75 ± 0.95
	IC_{50} (μM)	4.59	3.45	2.75	1.26	6.67
6	10 μM	61.13 ± 2.35	72.68 ± 0.79	80.22 ± 2.10	76.84 ± 1.24	61.03 ± 2.82
	30 μM	82.84 ± 0.92	89.08 ± 0.94	87.03 ± 1.45	81.08 ± 1.72	78.09 ± 1.79
	IC_{50} (μM)	7.48	4.24	2.98	4.65	1.46
7	10 μM	30.94 ± 2.42	39.34 ± 0.86	34.70 ± 1.42	n.d.	n.d.
	30 μM	68.82 ± 1.68	57.70 ± 0.75	52.69 ± 3.13		
	IC_{50} (μM)	n.d.	n.d.	n.d.		
Cisplatin	10 μM	20.84 ± 0.81	53.03 ± 2.29	51.00 ± 2.02	67.51 ± 1.01	60.25 ± 3.31
	30 μM	74.47 ± 1.20	86.90 ± 1.24	57.95 ± 1.45	87.75 ± 1.10	61.92 ± 1.01
	IC_{50} (μM)	19.13	5.78	9.78	3.74	6.19

n.d.: not determined.
[a] IC_{50} values were calculated from two experiments with 5 parallel wells if the growth inhibition of the compound was > 75% at 30 μM.

antiproliferative agents against various breast cancer cell lines. The calculated IC_{50} values representing their growth-inhibitory effects are presented in Table 1. As shown by our results, the configuration of the substituents at positions 16 and 17 play a limited role in the agents' antiproliferative properties. On the other hand, substituents, especially the *p*-methoxy group on the benzyl function at C-3 may enhance the anticancer potency. The compounds were additionally tested against the non-cancerous MRC-5 cells in order to obtain data concerning their selectivity towards cancer. In this regard, our compounds were not selective towards cancer cells but they were comparable to the reference agent cisplatin. Based on these results the analog with the lowest IC_{50} value (**5**) and its related, unsubstituted analog (**1**) were selected for additional investigations.

3.2. Cell cycle analysis by flow cytometry

In order to characterize the mechanism of action of the selected compounds against the MDA-MB-231 cell line, flow cytometry was carried out and the cell cycle phase distributions were analyzed after 24 h of incubation. The applied concentrations were determined based on the calculated IC_{50} values. Both compounds elicited a significant and concentration-dependent increase of the cell population in the G1 phase, on the expense of the reduction of those in the G2/M phase (Fig. 2). In spite of its substantially higher calculated IC_{50} **1** elicited remarkable cell cycle disturbance even at 5 μM. Additionally, **1** and **5** induced a modest but significant elevation of the hypodiploid (subG1) cell population at all the applied concentrations indicating the proapoptotic potential of these compounds.

3.3. Wound healing assay

As another means of investigating the anticancer activity of the tested estradiol derivatives, the possible motility inhibiting properties were examined by a wound healing assay. Migration of untreated MDA-MB-231 cells within the wound of the chambers was considered as 100% after 24 h incubation. Treatment with **1** and **5** significantly reduced the movement ability of MDA-MB-231 cells even at sub-antiproliferative concentrations (Fig. 3).

3.4. Boyden chamber assay

Besides the anti-migratory effect, the influence on the invasion

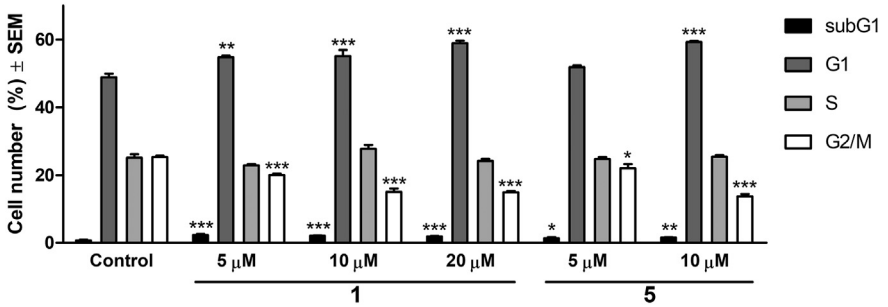

Fig. 2. Effects of compounds **1** and **5** on the MDA-MB-231 cell cycle after incubation for 24 h. Results are mean values ± SEM from three measurements. *, ** and *** indicates $p < 0.05$, $p < 0.01$ and $p < 0.001$ as compared t with the control cells, respectively.

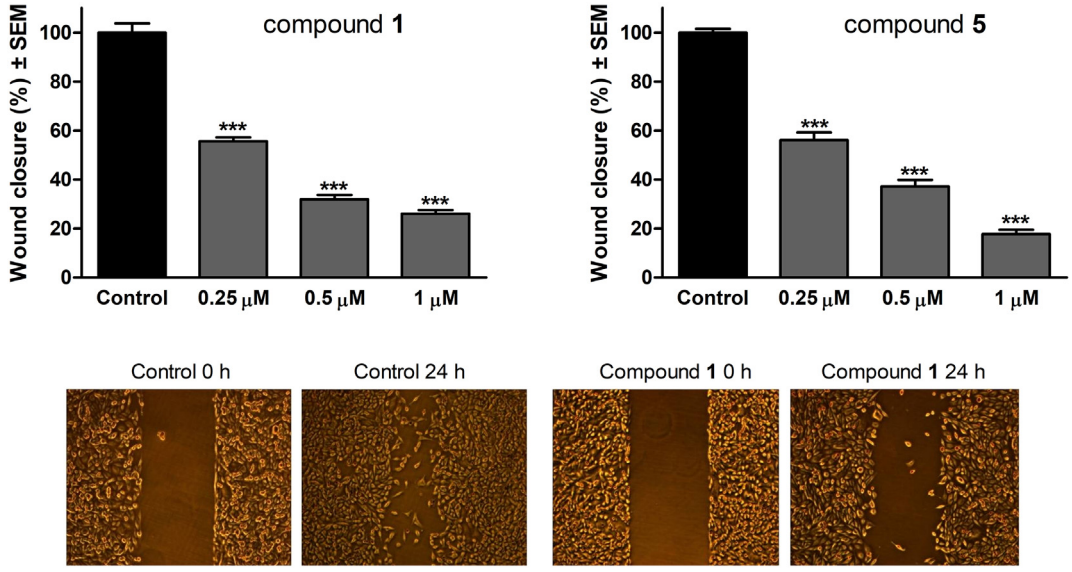

Fig. 3. Effects of compounds **1** and **5** on the migratory capacity of MDA-MB-231 cells (upper panels). The rates of the migration were evaluated after incubation for 24 h. Results are mean values ± SEM from three measurements *** indicates $p < 0.001$ as compared with the untreated control samples. Representative images of the migrating cells in 0 and 24 h (lower panels).

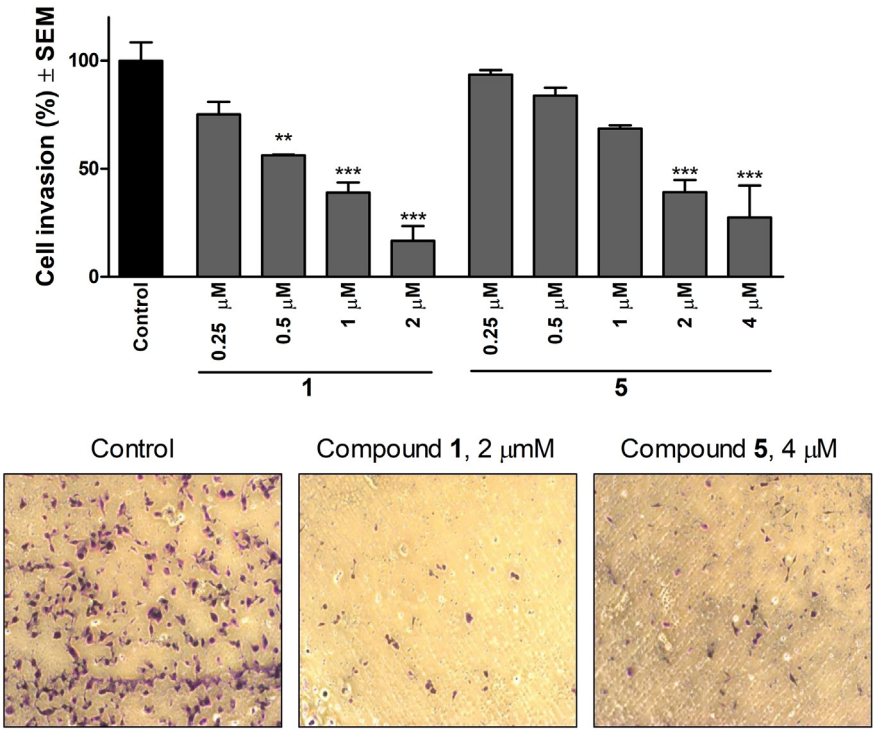

Fig. 4. Effects of compounds **1** and **5** on the cell-invasion capacity of MDA-MB-231 cells (upper panels). Statistical analyses of the percentage of invasive cells after incubation for 24 h with the test compounds. Results are mean values ± SEM from three measurements. *, ** and *** indicates $p < 0.05$, $p < 0.01$ and $p < 0.001$ as compared with the untreated control samples, respectively. Images from a visual field of one representative experiment (lower panels).

capacity of tumor cells is another important descriptor of the anti-metastatic property. The Matrigel Matrix-coated membrane (8.0 μm pore diameter) in Boyden chambers allows invasive cells to cross the membrane while it blocks the migration of non-invading cells. The invasion of MDA-MB-231 cells was found to be blocked by the compounds tested, in a concentration as low as 0.5 μM of **1** and 2 μM of **5**, and the effect size increased with higher concentrations (Fig. 4).

3.5. Gelatin zymography assay

In order to find a possible explanation for the metastasis-inhibitory effect of compounds **1** and **5**, the activities of released MMP-2 and MMP-9 were determined by a gelatin zymography assay. The active enzymes break down gelatin leaving a light patch on the blue background. None of our compounds exerted a significant influence on the activity of either MMP-2 or MMP-9 up to the concentrations of 2 μM (**1**) or 4 μM (**5**), suggesting that the antimetastatic activity of the tested compounds are independent of these enzymes (Fig. 5).

3.6. Western blot assay

The inhibition of focal adhesion kinase (FAK) and its phosphorylated form (pFAK) is another possible mechanism to block metastatic processes. Therefore the changes elicited by the tested compounds in the levels of proteins FAK and pFAK proteins were determined. Both compounds **1** (0.5–2 μM) and **5** (1–4 μM) reduced the level of phospho-

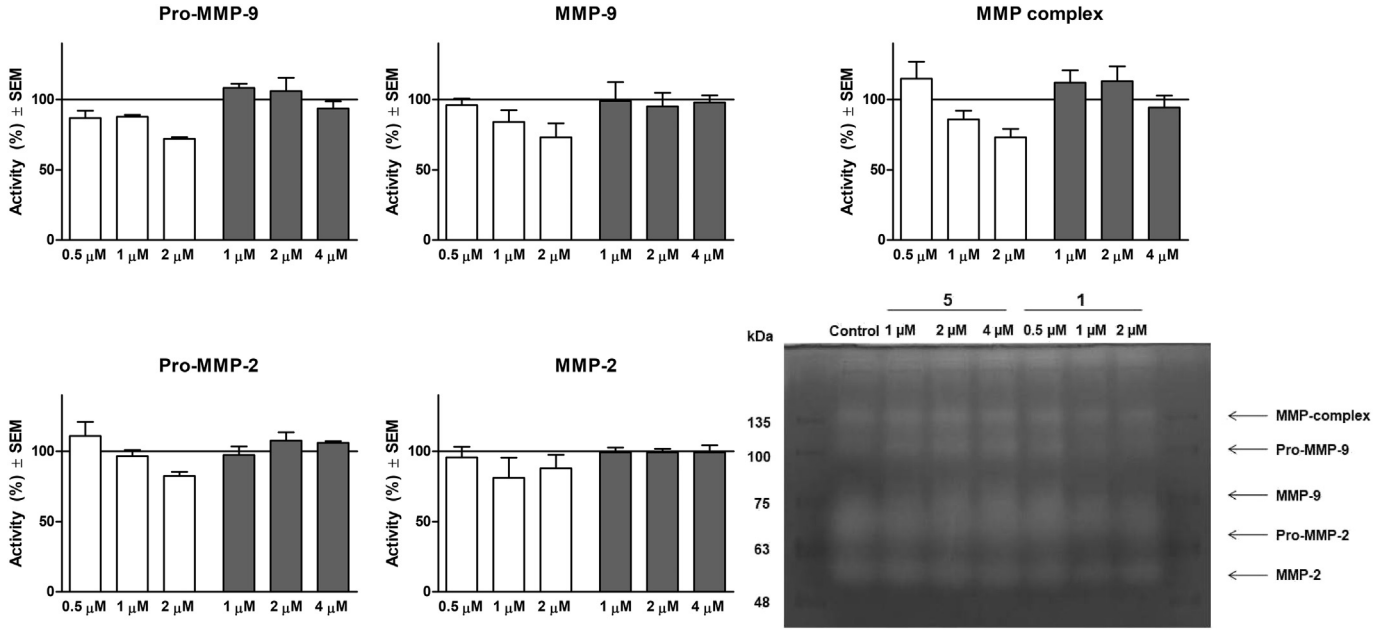

Fig. 5. Effects of compounds **1** and **5** on the activities of MMP-9 (pro-MMP-9, MMP-9 and its complex form) and MMP-2 (pro-MMP-2, MMP-2). Results are mean values ± SEM from three measurements. The activities presented in graphs are expressed as the % of vehicle control group. None of the treatment-related differences are significant. A representative zymogram (right bottom panel).

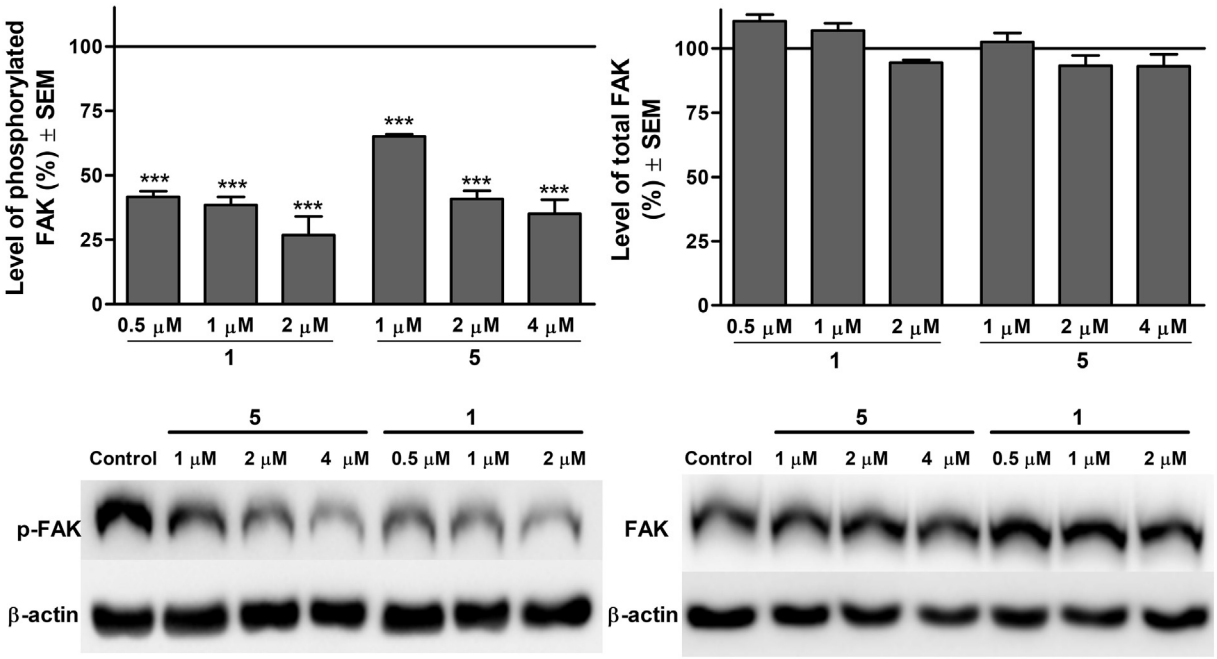

Fig. 6. Effects of compounds **1** and **5** on the expression of phosphorylated and total FAK protein in MDA-MB-231 cells (upper panels). Results are mean values ± SEM from three measurements. *** indicates $p < 0.001$ as compared with the untreated control samples. Representative results of the Western blot analysis (lower panels).

FAK in a concentration-dependent manner, without exerting any action on the total amount of FAK, indicating that the phosphorylation of FAK protein is inhibited by the compounds tested (Fig. 6).

4. Discussion

An endogenous derivative of 17β-estradiol (E2), 2-methoxyestradiol (2-ME) was discovered as the first estrane analog with potent growth-inhibitory effect, but without binding to the estrogen receptor (ER) (LaVallee et al., 2002). Since the possible mechanism of action, pharmacokinetic properties, selectivity and toxicity of 2-ME have been widely investigated, potential antiproliferative agents with an estrane skeleton are a new focus of the research and development of anticancer drug candidates (Kumar et al., 2016). Several studies have demonstrated that the antitumor mechanism of action of 2-ME comprises antiproliferative, antitubulin, antiangiogenic, antimetastatic and proapoptotic effects (Minorics and Zupkó, 2018; Pribluda et al., 2000; Zhu and Conney, 1998). Growth inhibition induced by 2-ME treatment in numerous malignant cell lines, including lung, colon, breast, prostate, eye, kidney, esophagus, stomach and pancreas cancers, as well as

tumors of the nervous system, melanoma, gynecologic malignancies, angiosarcoma and carcinoma of the musculature, was confirmed. Amongst the examined cell lines, breast carcinoma was found to be the most sensitive against 2-ME (Mueck and Seeger, 2010). It has been reported that its antiproliferative action against various cancer cell lines is mainly based on inducing blockade in the G2/M phase of cell cycle in various cancer cell lines (Carothers et al., 2002; Li et al., 2004; Pribluda et al., 2000; Qadan et al., 2001).

Treatment with 2-ME resulted in mitotic arrest in the prometaphase through the intense early up-regulation of cyclin B1 and Cdc2 proteins in both ER-positive and negative breast cancer cell lines. A close correlation was observed between the time-dependent variations of the prometaphase arrest and the level of the G2/M population. These results suggest not only the potent anti-microtubule effect of 2-ME but also demonstrate that this action is not influenced by the presence of estrogen receptor (Choi and Zhu, 2012). Despite 2-ME was well-tolerated in clinical trials, both as monotherapy and in combination with other anticancer agents, its activity was modest, even in a NanoCrystal Dispersion (NCD) formulation possessing more advantageous bioavailability (Harrison et al., 2011; Kumar et al., 2016; Matei et al., 2009). Despite this failure these studies showed that the modification of the estrane skeleton may yield promising anticancer agents. The structure of 2-ME has been recently utilized to design and synthesize a set of quinazolinone-based microtubule disruptors indicating the unexploited potency of the estrange skeleton as a base for innovative anticancer agents (Dohle et al., 2018).

Natural 17β-estradiol has been recently demonstrated to bind to DNA directly and that binding is not limited to the estrogen response element but it can affect random DNA sequences. This binding involves the intercalation of the steroid between base pairs of DNA, forming aromatic interactions with these base pairs (Hilder and Hodgkiss, 2017). These findings may serve as an explanation for the hormonal receptor independent character of a wide range of estrogen-related potential antiproliferative molecules including our present analogs.

Since the requirements of binding to the estrogen receptors are well-established, it is also known that a methlyl substitution at C-3 by itself is sufficient for the loss of the estrogenic activity (Anstead et al., 1997). Another opportunity to configure estrogen derivatives with low receptor binding affinities (RBA) is the specific substitution at C-16 with a polar or large group, such as hydroxymethyl. The compounds we examined contain a benzyloxy function at C-3 and a hydroxymethyl at C-16, therefore the lack of hormonal activity can be expected. This feature of the isomers of 16-hydroxymethyl-3,17-estradiol analogs was confirmed by radioligand-binding assays (Tapolcsányi et al., 2002). Previously we have reported on the antiproliferative action of estradiol-based compounds containing several distinct modifications at C-3 and C-16. The 16-triazole compounds of the 13α-estrone series were found to be effective against seven different cancer cell lines (Mernyák et al., 2015). Furthermore, a set of 16-hydroxymethyl-3-methoxy- and 16-hydroxymethyl-3-benzyloxy-13α-estradiol derivatives were demonstrated to exert potent anticancer activity against several gynecological cell lines (Kiss et al., 2018).

These previous accomplishments have encouraged us to continue the experiments with estradiol-derivatives, leading to the examination of the growth-inhibitory potential of a set of 16-hydroxymethyl-estradiols against various human adherent breast cancer cell lines.

Based on our data, the configurations of the substituents at positions 16–17 have a limited role in the agents' antiproliferative potency, while the benzyloxy function with *p*-substituent at position C-3 enhance the antiproliferative effect. Concerning the antiproliferative activities of the tested agents compound **5** proved to be the most potent and in terms of acting on non-cancerous cells it was comparable with cisplatin, which is currently used in breast cancer therapy protocols (Carey, 2015).

Although **5** and its precursor molecule **1** showed appreciable growth-inhibitory effects against all of the cell lines examined (MCF-7, MDA-MB-231, MDA-MB-361, T47D), our primary purpose was to characterize its effects and mechanism of action against MDA-MB-231, a triple-negative breast tumor cell line. In spite of the different receptorial status of the utilized cancer cell lines the IC_{50} values determined for a given agent were not crucially different. The aim of our study was to describe antiproliferative actions which are unrelated to receptorial status and our experimental system was optimized for this purpose. The medium contained phenol red which has some estrogenic action and it was also supplemented with usual fetal bovine serum instead of charcoal stripped fetal bovine serum which contains no residual steroids.

Performing cell cycle analyses by flow cytometry revealed that treatment with these agents caused G1 phase accumulation with significantly elevated apoptotic cell population. These results are in line with previously described G1 blockades by estradiol-related compounds such as two unsubstituted 16-oxime estrone analogues and oxadiazole derivatives of estrone (Berényi et al., 2013; Mernyák et al., 2013).

Approximately 90% of human cancer deaths are related to cancer metastases (Mehlen and Puisieux, 2006). This multi-step process involves local migration and invasion of tumor cells into adjacent tissues, entering into the vasculature, surviving and leaving the circulation system, with subsequent proliferation in distant organs that leads to colonization (Eger and Mikulits, 2005). Therefore, besides the cytotoxic and apoptosis-inducing approaches currently utilized in oncotherapy, innovative drugs with an antimetastatic capacity are eagerly needed to control cancer-related mortality. Besides their antiproliferative activity, the compounds tested in our experiments also inhibited cell migration and invasion, the initial steps of metastatic spread. Moreover, this significant reduction in cell migration was observed at a sub-antiproliferative concentration as low as 0.25 μM, after treatment for 24 h. The inhibitory action of compound **1** on cell invasion capacity proved to be more pronounced as determined by the Boyden chamber assay.

The role of matrix metalloproteinases (MMPs), a family of zinc-dependent endopeptidases have been extensively investigated, since their expression and activity are increased in almost all human cancer compared with normal tissue (Egeblad and Werb, 2002). The increased expression of MMP-2 and MMP-9 at the mRNA level was demonstrated in MDA-MB-231 cells compared to normal HS578Bst breast cells. Moreover, high expression of MMP-2 and MMP-9 in cancerous breast tissues has a close correlation with lymph node metastasis and tumor staging (Li et al., 2017). Based on our results of the gelatin zymography assay, none of the examined compounds influenced substantially the level of MMP-2 and MMP-9, indicating that these metalloproteinases are not crucially involved in the mechanism of action of **1** and **5**.

Focal adhesion kinase (FAK) is expressed in most cell types and tissues and has a complex function in various processes including cell spreading, differentiation, cell cycle, migration and cell death. The activation of FAK principally occurs via autophosphorylation on tyrosine 397 by clustering of integrins (McLean et al., 2005). Several studies have demonstrated its role in the regulation of cell migration. The up-regulation of FAK was reported in various cancer types, including malignancy of the breast, and its elevated activity correlates with metastatic spread and poor prognosis of the disease (Miyazaki et al., 2003; Recher et al., 2004). In breast cancer, FAK overexpression is regarded as an early event in tumorigenesis which precedes cell invasion and metastasis in ductal carcinoma in situ (DCIS) (Lightfoot Jr. et al., 2004). On the contrary, the lack of FAK-activity is reported to result in poor cell migration in response to chemotactic and haptotactic signals, while overexpression of FAK increases cell migration in Chinese hamster ovary (CHO) cells (Parsons et al., 2000). Our present compounds decreased the level of pFAK (Y397) in a concentration-dependent manner, whereas total FAK level was unaffected, demonstrating that the phosphorylation of the kinase enzyme was inhibited. These are the first reported estrogen-related compounds which proved to be effective in the inhibition of FAK phosphorylation.

In summary, our study revealed that 3-substituted C16-hydroxymethyl-estradiols are potent antiproliferative agents against breast

cancer cell lines. In MDA-MB-231 triple-negative cell lines 2 test compounds caused programmed cell death and G1 phase elevation. Furthermore, the molecules significantly and concentration-dependently inhibited cell migration and invasion, as well as the phosphorylation of focal adhesion kinase in sub-antiproliferative concentration. These results indicate that modified estradiol-derivatives can be regarded as promising candidates in the design of new anticancer agents.

Conflict of interest

The authors have no conflict of interest to declare.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejps.2018.07.029.

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